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# Suppression of mutagenesis by Rad51D-mediated homologous recombination

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## SUMMARY

Homologous recombinational repair (HRR) restores chromatid breaks arising during DNA replication and prevents chromosomal rearrangements that can occur from the misrepair of such breaks. In vertebrates, five Rad51 paralogs are identified that contribute in a nonessential but critical manner to HRR efficiency. We constructed and characterized a Rad51D knockout cell line in widely studied CHO cells. The *rad51d* mutant (51D1) displays sensitivity to a wide spectrum of induced DNA damage, indicating the broad relevance of HRR to genotoxicity. Untreated 51D1 cells exhibit ~5-fold elevated chromosomal breaks, a 12-fold increased rate of *hprt* mutation, and 4- to 10-fold increased rates of gene amplification at the *dhfr* and *CAD* loci, respectively. These results explicitly show the quantitative importance of HRR in preventing these types genetic alterations, which are associated with carcinogenesis. Thus, HRR copes in an error-free manner with spontaneous DNA damage encountered during DNA replication, and Rad51D is essential for this fidelity.

## INTRODUCTION

A major goal in cancer biology is to understand the mechanisms of origin of genomic alterations that promote the progressive conversion of normal cells to a fully malignant phenotype. Double-strand break (DSB) repair pathways play a pivotal role in carcinogenesis, as chromosomal rearrangements are a fundamental feature of cancer cells. In model systems, mutations in DSB-repair pathways consistently manifest phenotypes of spontaneous chromosomal instability as elevated aneuploidy, chromosome breaks and exchanges, and micronuclei. However, there is little information on the contributions of the DSB pathways to spontaneous genetic alterations that are inherently compatible with cell viability.

Deleted: carcinogenesis

The DNA repair pathways of nonhomologous end joining (NHEJ) and homologous recombinational repair (HRR) are responsible for eliminating double strand breaks (DSBs) arising from endogenous processes or DNA damage caused by exogenous agents. In mammalian cells HRR is critical for restarting broken replication forks that encounter single-strand breaks or other lesions (18, 56), and for the error-free repair of DSBs occurring in chromosomal regions that have already replicated during S phase. HRR is essentially inactive during G1 phase since DSB-mediated recombination between homologous chromosomes occurs at a very low frequency ( $10^{-5}$  to  $10^{-6}$ ) (37). HRR activity is responsible for the classical S-phase resistance of cells to ionizing radiation and may decline in G2 phase (21, 38).

In vertebrate cells, HRR is mediated by the Rad51 strand transferase acting with other proteins that include the five Rad51 paralogs (XRCC2-3, Rad51B-C-D), as reviewed in (54, 57). Although cycling cells die rapidly without Rad51 (47), the ancillary function(s) provided by the Rad51 paralogs are not essential as mutants can grow, but with impaired viability. Mutant cell lines defective in *XRCC2*, *XRCC3*, or *RAD51C* were produced by random mutagenesis in

Chinese hamster cells, isolated on the basis of IR sensitivity (13, 23), and used to clone the complementing human cDNAs (5, 12, 16, 26). These mutants consistently show high levels of chromosomal aberrations, extreme sensitivity to crosslinking agents, modest sensitivity to ionizing radiation (IR). They also show defective IR-induced Rad51 focus formation and defective HRR measured by the repair of an enzymatically induced DSB in a direct repeat substrate (57). Although the hamster cell HRR mutants have proved valuable, none is isogenic with its parental cell line because of induced mutagenesis, and they are often not fully complemented by transfected human cDNAs or genes. In chicken DT40 cells, gene targeting has produced mutants for all five paralogs (50, 51). Although they share some common phenotypic traits and resemble the hamster mutants in many respects, among them they have differential sensitivity to the crosslinker cisplatin and the DSB-induced agent camptothecin (62).

Gene knockouts of *Xrcc2*, *Rad51b*, and *Rad51d* in mice cause embryonic lethality, usually early in development (8, 36, 42). In the *Xrcc2* mouse knockout study (9), early growth arrest of MEFs from 13.5-day-old mutant embryos in culture was observed. However, it was possible to obtain immortalized MEF cultures at a very low frequency. Both primary and immortalized MEFs displayed substantial gain and loss of chromosomes in addition to elevated chromosomal aberrations (9). Using the *rad51d* knockout mouse, MEF cell lines could be established in a Trp53-deficient background, and these cells exhibited chromosomal instability, aneuploidy, and centrosome fragmentation, but no reduction in spontaneous sister-chromatid exchange (SCE) (46). *XRCC3* was knocked out in the HCT116 human tumor cell line, and this mutant has a much weaker phenotype (63) compared with the *xrcc3* mutants of rodent and chicken cells. HCT116 and other mismatch repair-defective tumor cells have reduced expression of the Mre11-Rad50-Nbs1 complex (14) and are, therefore, inherently defective in HRR (61).

In this study we describe a new isogenic *rad51d* mutant of CHO cells and characterize its phenotype, with emphasis on spontaneous genetic instability. As expected, we find greatly enhanced spontaneous chromosomal breakage and exchange although, paradoxically, SCE is unchanged. Most importantly, we demonstrate increased spontaneous gene amplification rates at the *CAD* and *dhfr* loci, as well as a greatly increased mutation rate at the *hprt* locus. We also confirm these findings with another Rad51 paralog CHO mutant, *xrcc3* irs1SF. These studies provide the first determination of the quantitative contribution of Rad51 paralogs in preventing these two classes of gene-specific alterations that are intrinsically relevant to carcinogenesis.

## MATERIALS AND METHODS

**Cell culture and cell cycle analysis.** CHO AA8 cells (55) were grown in monolayer or suspension culture in  $\alpha$ MEM supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin. Cells were counted and analyzed on a Coulter® Multisizer II. The plating efficiency of AA8 and other repair-proficient cell lines was ~90%, and that of 51D1 was ~70%; the doubling times for AA8 and 51D1 were ~13 h and ~16 h, respectively.

To determine the cell cycle distribution of each cell line  $5 \times 10^5$  cells were treated with 10  $\mu$ g/ml BrdUrd for 20 min at 37°, fixed with 70% ethanol, and stained with FITC-conjugated anti-BrdUrd antibody (BD Biosciences) and propidium iodine to determine the proportion of cells in each phase and DNA content, respectively. Fluorescence measurements of each sample were made on a FACScan (Becton Dickinson) and the data analyzed using Cell Quest software.

**Mutagen sensitivity.** Mutagen sensitivity was determined by colony formation in 10 cm dishes. When most colonies were clearly visible by eye, dishes were rinsed with PBS, fixed with 95% ethanol and stained with Gram Crystal Violet (Becton Dickinson). Exposure to genotoxic

agents was as follows: UV radiation, as described (55);  $^{137}\text{Cs}$   $\gamma$ -irradiation, at  $5 \times 10^5$  cells in 15-ml tubes kept on ice; MMS and MMC, at  $1 \times 10^6$  cells in 10 ml suspension cultures were exposed to drug at 37°C for 60 min, chilled on ice, centrifuged, resuspended in fresh medium.

**Cytogenetic analyses.** Mitotic cells were collected by a shake-off procedure, obviating the need for colcemid collection, and centrifuged at 1000 rpm for three min. The cell pellet was gently broken up, resuspended in 10 ml of 37°C 75 mM KCl hypotonic buffer, and incubated for 7 min in a 37°C water bath. Two ml of fresh 3:1 methanol:acetic acid (Carnoy's) fixative was added directly to the cell suspension in hypotonic buffer and gently mixed. The suspensions were centrifuged at 1000 rpm for four minutes, the supernatant was removed and the cell pellet was gently broken up and fixed dropwise with 4 ml of fresh fixative. This procedure was repeated two more times, and cell suspensions were dropped onto cold, wet slides, air-dried and desiccated for 24 h at 37°C. The next day, slides were stained in a 10% Giemsa solution (Gurr), dried and mounted with CytoSeal™ 60 mounting medium (Microm International) and a coverslip. Non-polyploid metaphase chromosome spreads of good quality were examined under a 100× objective and 2× optivar using a Nikon Microphot microscope. Chromosome numbers and chromosome aberration frequencies (mainly chromatid-type) were scored. Chromatid gaps were defined as fully achromatic lesions less than the width of a chromatid arm, chromatid breaks being separated at a width greater than the chromatid arm or displaced from the main chromatid axis (40). Sister chromatid exchange measurements were performed as described previously (30) by measuring 50 cells for each cell line in each of two experiments.

**Rad51 targeting vector construction.** Gene-targeting vectors pTnT-neo.LARA and pTnT-puro.LARA were derived from a universal backbone targeting vector designated pTnT.neo

(details available on request). pTnT-neo.LARA was constructed by first inserting a 285 bp PCR fragment containing *RAD51D* exon 4 between *XhoI* and *AflIII* of pTnT-neo. A 2.8 kb *HincII/HindIII* blunted-end fragment of the left arm containing the sequences of *Rad51D* intron 3 region was ligated into pTnT-neo at the *BstEII* site to create a 9.1 kb pTnT-neo.LA. The right arm containing a 2.7 kb *KpnI/XmnI* blunted-end fragment from regions of exons 5 & 6 and introns 4-6 was inserted into pTnT-neo.LA at the filled-in *AscI* site to create the 11.8-kb pTnT-neo.LARA construct used to target the first *RAD51D* allele. The left arm of the gene-targeting vector containing the puromycin gene was created by first ligating a 4.2 kb *BamHI/PacI* fragment of pTnT-puro and a 5.0 kb *BamHI/PacI* fragment from pTnT-neo.LA to produce a 9.2 kb pTnT-puro.LA. A 335 bp *AscI/XhoI* fragment containing exon 4 derived from pTnT-neo.LARA was cloned into the *AscI/XhoI* sites of pTnT-puro.LA, and the 2.7 kb *AscI/ClaI* right arm fragment was inserted into the modified pTnT-puro.LA at the *AscI/ClaI* restriction sites containing exon 4 to create the 12.1 kb pTnT-puro.LARA, which was used to disrupt the second *RAD51D* allele.

**DNA transfection.** For gene targeting,  $3 \times 10^7$  cells were washed and resuspended in 1 ml cold electroporation buffer (20 mM HEPES (pH 7), 137 mM NaCl, 5 mM KCl, 0.7 mM  $\text{Na}_2\text{HPO}_4$ , 6 mM glucose), mixed with 10  $\mu\text{g}$  linearized (pTNT.neo.LARA or pTNT.puro.LARA) DNA, electroporated at 260 V/1600  $\mu\text{F}$ , incubated for 5 min on ice, and plated in T150 flasks for 24 h to allow for *selective marker* expression (see below).

**Targeting *RAD51D* alleles.** Cells transfected with pTNT.neo.LARA were plated into 10-cm dishes at  $\sim 2 \times 10^6$  cell/dish in 20 ml medium containing 1.7 mg/ml G418 (Gibco Invitrogen) and incubated for 5 days at 5%  $\text{CO}_2$  and 37°C, after which the medium was replaced with fresh medium (supplemented

with 10% dialyzed serum) containing 0.1  $\mu$ M 2'-fluoro-2'-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil (FIAU); cells were incubated an additional 5 days. Each dish contained a pool of ~150 drug-resistant colonies, which were harvested for freezing and DNA isolation (QIAamp® DNA Blood Mini Kit, Qiagen Inc). The frequency of G418 resistant colonies averaged  $2 \times 10^{-4}$ , and the FIAU enrichment was ~6.3-fold. A single pool of clones containing a targeting event was processed through two successive rounds of PCR screening, first in 30 sub-pools of 15 clones each, and then as single clones. Transfectant pools were screened by PCR in three steps. First, we tested for HR of the left arm of the targeting vector by PCR amplification from *neo* to upstream *Rad51D* sequence. Second, pools were tested by PCR across the right arm from *neo* to downstream *Rad51D* sequence, and finally across the entire targeted (extended) gene. The experiment yielded at least 3 independent clones (one electroporation yielded three positive dishes). One positive clone was then used to target the second *RAD51D* allele by transfecting with pTNT.puro.LARA and selecting in 10.5  $\mu$ g/ml puromycin for two days. Selection media was changed to G418/FIAU medium at 1.7 mg/ml and 0.1  $\mu$ M, respectively (10% dialyzed serum) and grown for 5 days. Selection in G418 ensured elimination of clones that retargeted the allele already targeted in the first round of targeting. Puromycin (10.5  $\mu$ g/ml) was then added back to the dishes and incubated an additional 4-5 days to ensure all killing of cells without puromycin integration. Each dish contained a pool of ~150 drug-resistant colonies. The frequency of puro-resistant colonies averaged  $\sim 5 \times 10^{-4}$ , and the FIAU enrichment was ~6.5-fold. Gene-targeted cells were identified and cloned as detailed above. Three clones independently targeted for the second allele were identified.

Two positive clones were treated with Cre recombinase plasmid, pBS185. We identified single clones that underwent Cre-mediated recombination between the LoxP sites flanking the targeted 51D exon 4 and selectable marker sequences. The identification was based on sensitivity to both selectable markers, as well as MMC.

**Western blotting.** Nuclear extracts were prepared from CHO cells using the NE-PER kit (Pierce Biotechnology) and separated on a 12% Bis-Tris gel (NuPage system, Invitrogen) after normalization for loading by Bradford analysis. After blotting to PVDF membrane, the blot was blocked overnight in PBS-T containing 5% milk at 4°C. Anti-Rad51D 5A8/4 (Novus Biologicals) was used at 1:300 for 2 h at room temperature in PBS-T/5% milk and washed with PBS-T before incubation with secondary antibody, anti-mouse-HRP (Santa Cruz Biotech) in PBS-T/5% milk. Final development was achieved using a chemiluminescent HRP substrate (BioRad). Antibody against Lamin A/C (H-110) (Santa Cruz Biotech) was used as a loading control, as described above at 1:300 with an anti-rabbit-HRP secondary (Santa Cruz Biotech).

**Rad51 immunofluorescence.** Ten ml of cell suspension at  $1 \times 10^5$  cell/ml was treated with 5  $\mu$ M MMC or 75  $\mu$ g/ml MMS for 1 hr, washed once with medium, and resuspended in 10 ml fresh medium. Cells were incubated for 4 h and then centrifuged onto glass slides at 2000 rpm for 5 min using a Cytospin® 4 cytocentrifuge (Thermo Shandon). Cells were fixed in 2% paraformaldehyde for 15 min, permeabilized in cold 0.2% Triton X-100 for 5 min, and blocked in 1% BSA for 1 hr. The slides were incubated with anti-Rad51 antibody (clone H-92, Santa Cruz Biotechnology) at 4°C overnight (1:1000 dilution in 1% BSA), and Alexa Fluor® 488 goat anti-rabbit secondary antibody (A-11008; Molecular Probes) at room temperature for 1 h. Glass slides were mounted using Vectashield mounting medium with DAPI (H-1200; Vector Laboratories). Fluorescence images were captured on Quips PathVysion using an Axiophot II fluorescence microscope and Rad51 foci were counted visually.

**Mutation and gene amplification rates.**

*Hprt* mutation rate and gene (*dhfr* and *CAD*) amplification rates were determined by fluctuation analysis (27) as detailed elsewhere (20).

## RESULTS

**Targeted conditional disruption of *RAD51D*.** The strategy for inactivating *RAD51D* was to delete exon 4 (amino acids 88-115), which contains the GKT Walker A-box for ATPase activity. This deletion also changes the reading frame and results in a highly truncated polypeptide (S88→R...114X). In order to disrupt the two alleles of *RAD51D* (as determined by FISH analysis; data not shown), alleles were targeted one at a time using two different selectable markers (Fig. 1A). Targeting vectors were designed to maintain functional *RAD51D* alleles such that both exon 4 and the associated selectable marker are flanked by LoxP sites. After transfection with the targeting vector containing the *neo* gene and selecting G418 resistant clones, pools of clones were screened by PCR analysis, using primers specific for DNA sequence in *neo* and genomic DNA sequence outside of the homologous arms in the vector, to determine the presence of correctly targeted cells. Positive pools were reduced to pure clones as detailed in Experimental Procedures, and such a clone was then transfected with the vector containing the *puro* selectable marker. Transfectants were selected in puromycin and G418 to insure the initial allele was not re-targeted. Pools of clones were screened by PCR analysis in the same manner as in the first-allele targeting. Two clones, from independent pools and having both *Rad51D* alleles targeted, were isolated and designated 51D1Lox and 51D2Lox. Simultaneous deletion of exon 4 in both alleles was accomplished by transfection with Cre recombinase, resulting in mutant clones 51D1 and 51D2, respectively. Disruption was verified by the inability of Cre-treated cells to grow in the presence of puromycin, G418, and MMC (Fig. 1B). Western analysis was performed to verify absence of the Rad51D protein in the knockout cells. Fig. 1C shows the absence of the 39-kDa band in the 51D1 cells. The level of Rad51D appears higher in the AA8 parental cells than in the 51D1Lox and the 51D1.3 gene-complemented cells. *RAD51D*

expression may be reduced in 51D1Lox cells because of the embedded *neo* and *puro* gene promoters, which may retard transcription. The 51D1.3 cells may possess only one copy of the gene, whose expression could also be influenced by its ectopic location.

Exponentially growing 51D1 cells show clear abnormalities in their cell cycle distribution (Fig. 1D). There is a much higher proportion of cells in both S and G2/M phases compared with the AA8 and 51D1.3 control lines. Moreover, there is a measurable increase in the proportion of tetraploid cells (5%) in 51D1 cultures. Each of these features is qualitatively similar to what was seen in *rad51d trp53* knockout mouse embryonic fibroblasts (46).

**Increased sensitivity of *rad51d* cells to killing by diverse DNA damaging agents.** We used colony formation assays to determine survival of *rad51d* cells after exposure to four commonly used and distinctly different DNA-damaging agents (Fig. 2). The *rad51d* 51D1 cells show exquisite sensitivity (~80-fold) to the interstrand crosslinking agent MMC relative to parental AA8 and 51D1Lox, and the gene-complemented clone 51D1.3 (Fig. 2A). (Fold sensitivity is measured as the dose-reduction factor at 37% cell level.) With  $\gamma$ -rays, both the 51D1 and 51D2 mutant clones show a ~1.5-fold sensitivity (Fig. 2B), similar to that of the *xrcc3* *irs1*SF mutant. 51D1 cells show substantial sensitivity (~5-fold) to the alkylating agent MMS (Fig. 2C), as well as ~2-fold sensitivity to UV-C (Fig. 2D). For each agent the mutant cells are fully complemented when they express the hamster *RAD51D* gene (clone 51D1.3). These results show that the impairment of HRR through loss of Rad51D compromises the ability of CHO cells to deal with very diverse types of DNA damages.

**Reduced Rad51 foci in *rad51d* cells after exposure to IR.** Lack of Rad51 nuclear focus formation after exposure to DNA-damaging agents is generally associated with HR deficiency in Rad51 paralog mutants of rodent and chicken cells [reviewed in (57)], including mouse *rad51d*

knockout cells (46). As expected, 51D1 cells exposed to 8 Gy  $\gamma$ -rays or 5  $\mu$ M MMS were grossly defective in Rad51 focus formation (Fig. 3A). Whereas the control cultures treated with  $\gamma$ -rays or MMS showed 62%-85% of cells with >5 foci per cell, 51D1 showed only 7% and 1% of cells with foci, respectively (Fig. 3B). In untreated cultures the 51D1 cells showed a less severe focus defect, i.e. an average of 2 foci per cell versus 3 foci per cell in the two control cell lines (data not shown).

**Increased spontaneous chromosomal aberrations in *rad51d* cells.** Chromosomal instability is a hallmark of HR-deficient cells. To determine the role of Rad51D in maintaining chromosomal integrity, we measured spontaneous chromosomal aberrations in a large population of cells. Relative to the Rad51D-proficient cell lines tested, the 51D1 cells showed a significant increase in the levels multiple types of aberrations (Table 1): chromatid breaks (5- to 12-fold), chromatid gaps (~3 fold), and isochromatid breaks (5- to 6-fold). Also seen in the 51D1 cells was a low level of chromatid exchanges, as illustrated in Fig. 3C, which were not detected in the Rad51D-proficient cells. Though dicentric chromosomes were no more prevalent in the 51D1 cells than in the parental 51D1Lox cells, ringed chromosomes were only detected in the 51D1 cells. Aneuploidy (chromosome gain or loss) has been associated with CHO and MEF cells deficient in the Rad51 paralogs XRCC2 and XRCC3 (9, 17). We wished to determine if the Rad51D deficiency caused a similar problem with maintenance of chromosome number. CHO AA8 cells have a modal chromosome number of 21 (10), which was maintained in both the 51DLox and 51D1 lines (Table 2). The percentage of cells having a gain or loss of one or two chromosomes from the modal value also remained constant. Furthermore, the Rad51D-complemented 51D1.3 cells maintained the same distribution of chromosome numbers. These findings suggest that aneuploidy is not associated with the *rad51d* mutation in CHO cells.

**Normal spontaneous sister-chromatid exchange (SCE) in *rad51d* cells.** As a classical, cytological manifestation of crossing over between sister chromatids, SCE could be expected to be reduced in 51D1 mutant cells. The average value from two experiments was  $0.30 \pm 0.1$  SCE per chromosome in each of the AA8, 51D1, and 51D1.3 cell lines. Thus, somewhat surprisingly, there was no detectable reduction in 51D1 cells.

**Increased spontaneous *hprt* mutation rate in *rad51d* cells.** An increased mutation rate at the *hprt* locus is a measure of genomic instability at the single-gene level. Using fluctuation analysis to measure mutation rates (27), we found that *rad51d* cells have a greatly increased rate (~12 fold) of *hprt* mutation calculated by both the  $P_0$  method and the method of the mean (4, 27) (Fig. 4). Importantly, the gene-corrected 51D1.3 cells and the pre-Cre-treated 51D1Lox cells had mutation rates like of that of the parental AA8 cells. Hypermutability was also seen with the *xrcc3* irs1SF cells, which had an even higher *hprt* mutation rate (~20-fold increased over AA8) than the 51D1 cells. 1SFwt8 cells, which express human *XRCC3* cDNA, were partially corrected for *hprt* mutagenesis (Fig. 4), as they were previously for cell survival after  $\gamma$ -irradiation and MMC treatment (60). The *aprt* locus in AA8 cells is heterozygous due to a point mutation at one of the two alleles (43, 55), and *aprt* is known to have ~10-fold higher mutation rate than *hprt* because of a high rate of deletion (1). There was no significant increase in mutation rate at the *aprt* locus in either *rad51d* or *xrcc3* cells. The *aprt* rate for AA8 was  $(10 \pm 3) \times 10^{-6}$  and for 51D1 was  $(13 \pm 4) \times 10^{-6}$ , based on the method of the mean. An increase in mutability at the *aprt* locus in the 51D1 cells could be difficult to detect, as the spontaneous rate is so high in AA8 cells that an increase comparable to that at *hprt* would be less than a doubling in the 51D1 cells.

**Increased spontaneous amplification rates in *rad51d* cells.** Gene amplification is another manifestation of genomic instability in which megabase regions of DNA are replicated in excess and maintained chromosomally or as extrachromosomal elements. The role of HRR in preventing such events is not known, but CHO cells deficient in NHEJ, because of mutant DNA-PKcs, have increased rates of amplification (29). We determined that the rate of gene amplification was increased 3- to 10-fold in HRR-deficient 51D1 and *irs1*SF cells at both the *dhfr* and *CAD* loci (Table 3). The gene-corrected mutant cell lines showed rates of amplification similar to those of the parental cells. To see if this amplification phenotype could be seen in another paralog mutant, we tested the V79-derived *xrcc2* cell line, *irs1*. It also displayed an increased amplification rate compared to the corresponding *XRCC2*-complemented cells (GT619) (26), consistent with a general role for the paralogs in preventing gene amplification.

## DISCUSSION

In this study, we created in CHO AA8 cells a knockout mutant of *RAD51D* that is designed to be isogenic with respect to AA8, 51D1Lox parental cells, and BAC gene-complemented 51D1 cells. To further ensure an isogenic relationship, the 51D1 mutant was complemented with the hamster *RAD51D* gene, rather than a human homolog as has often been done historically. Although the level of Rad51D expression in 51D1.3 cells appears to be lower than in AA8 cells (Fig. 1C), the level is adequate for full complementation of all aspects of the mutant phenotype. 51D1 is the first isogenic mutant in DSB repair to be constructed in CHO cells.

**Comparison of the phenotype of 51D1 cells with other Rad51 paralog mutants.** The *rad51d* CHO cells resemble *rad51d* knockout mouse MEFs in showing very high sensitivity to MMC and lesser sensitivity to IR, UV-C, and MMS (46). This common phenotype emphasizes

the importance of HRR in helping cells cope with a broad range of DNA lesions, all of which likely lead to a requirement during S phase for recombination to efficiently negotiate the lesions. A likely common form of damage is the single-strand break, produced either directly by the DNA-damaging agent (e.g. IR) or as an intermediate during repair (e.g. MMS and MMC), resulting in one-sided DSBs when encountered by the replication fork. Another theoretical possibility is a role for HRR in bypassing damaged bases in an error-free manner through a process of fork regression/reversal followed by restart of the replication fork (i.e. “chickenfoot” intermediate) (19).

The profile of sensitivity of the *rad51d* CHO cells to various DNA-damaging agents is also similar to that seen previously for hamster cells deficient in other Rad51 paralogs: *XRCC2* (irs1), *XRCC3* (irs1SF), and *RAD51C* (irs3) cells (12, 26, 53). The 80-fold sensitivity of 51D1 cells to MMC is dramatically higher than the ~3-fold sensitivity of the DT40 *rad51d* mutant to MMC (51). Similar differences have been seen between systems for other Rad51 paralog mutants, emphasizing that there are inherent differences between these model systems. The similarities among these Rad51 paralog mutants within each system suggest that the paralogs have a common role in HRR.

**Elevated gene amplification rates associated with HRR deficiency.** Our emphasis in this study was in characterizing spontaneous genetic instability. Gene amplification, a form of genetic instability in which large regions of genomic DNA are multiply replicated and maintained in the genome, results in tumor cells having increased copies of oncogenes and multi-drug resistance phenotypes (reviewed in (39)). DNA damage appears to play an important role in promoting gene amplification, as it is enhanced when cells are exposed to agents that break DNA (i.e.  $\gamma$ -rays and hydrogen peroxide) (28). Defects in DNA repair systems have previously been associated

with increased rates of gene amplification, including NHEJ deficiency in CHO cells (29) and mismatch repair deficiencies in human tumor cells (6, 25). We recently showed increased gene amplification rates in a *fancg* CHO knockout mutant defective in the Fanconi anemia chromosome stability pathway (20). As shown in Table 3, we have made the novel observation that HRR also plays an important role in preventing gene amplification as evidenced by the highly elevated rates in *rad51d* cells, and confirmed in the *xrcc3* *irs1*SF and *xrcc2* *irs1* mutants. However, the highest reported rates of *CAD* amplification occur in *dna-pkcs* cells, in which the elevation was 20- to 150-fold (29). From these collective findings it appears that multiple DNA repair pathways, including HRR, contribute to the prevention of gene amplification.

**Normal rate of SCE in *rad51d* cells.** Sister chromatid exchange, which is thought to be a cytological manifestation of HRR, occurs only a few times during each S phase. SCE is greatly increased by a broad range of genotoxic agents including inhibitors of DNA replication (22, 35). Evidence that SCE is caused at least in part by HRR was presented in chicken cells (48). However, models have also been proposed in which fork breakage and rejoining by NHEJ could be responsible (22, 33). SCE can presumably occur when broken replication forks are restarted by HRR (18). A priori, one might expect that the *rad51d* deletion would partially suppress SCE, but we found that 51D1 cells had no change compared with control cell lines. Although the *rad51d* mutant and other Rad51 paralog mutants in chicken DT40 cells have 2- to 3-fold reduced rates of SCE (51), mouse *rad51d* *trp53* knockout MEFs also show no reduction compared with the *trp53* control cells (46).

These findings of normal SCE rates in mammalian *rad51d* mutants have several possible explanations. First, Rad51D may have no role in the putative HRR exchange event associated with restarting a broken replication fork through the processing of a Holliday junction (HJ)

intermediate (18). This interpretation was put forth to explain the mouse *rad51d* MEF data (46). However, given the very high levels of spontaneous chromatid breaks, which most likely derive from unrepaired broken replication forks, this explanation seems unlikely. Second, SCE in wild-type cells might arise primarily from NHEJ by a process in which both parental strands are broken and forks are restored by rejoining parental strands with daughter strands. However, the normal frequency of SCE in *xrcc5/ku80* CHO mutants argues against this possibility (7). Third, the restart of broken forks by HRR may normally occur primarily through the “crossover” mode of HJ resolution, which would not produce SCE as cytologically identifiable events (18). The visible SCE could arise in a different manner, e.g. “non-crossover” mode of HR resolution, perhaps independently of Rad51D. In any event, other studies show that the loss of Rad51 paralogs in mammalian cells has only a modest or no influence on spontaneous SCE. Neither *xrcc2* *irs1* nor *xrcc3* *irs1* SF shows a significant reduction in SCE (13, 59), and *xrcc2* knockout mouse cells (both primary and immortalized) show only a 30% reduction (9). Two *rad51c* V79 mutants have a slightly reduced rate of spontaneous SCE (12, 16). Thus, mammalian Rad51 paralogs differ from the chicken homologs in having a lesser quantitative contribution to the rate of SCE. However, since the *rad51d* mutant MEF cells have a clear deficiency in MMC-induced SCEs (46), the mechanistic details of induced SCEs must differ from spontaneous events.

**Role of Rad51D in chromosome stability.** We observed substantially elevated chromosomal aberrations in *rad51d* cells, particularly chromatid and chromosome breaks, which presumably arise from broken replication forks that remain unrepaired. An essential role for Rad51D in telomere maintenance also has been reported for telomerase-deficient cells. Rad51D was shown to localize at the sites of telomeres in HeLa cells, and *rad51d trp53* MEFs have a high level of telomere end-to-end fusions as well as chromatid breaks and other chromosomal

abnormalities (46, 52). The association of Rad51D with telomeric sequences appeared to be specific compared with other Rad51 paralogs. In addition, Rad51D-deficient human cells exhibited telomeric DNA repeat shortening (52). CHO and other immortalized Chinese hamster cells lack cytologically visible telomeres (44), but they express telomerase activity (45) and exhibit interstitial telomeric bands that are subject to amplification (34). The absence of a requirement for Rad51D in telomere maintenance in CHO cells may explain why our *rad51d* mutant cells grow relatively robustly compared with the *rad51d trp53* mouse cells. Importantly, 51D1 cells show only a mild growth retardation, having a doubling time of ~16 h compared with ~13 h for 51D1Lox and AA8 cells.

**High mutability of Rad51 paralog mutants.** Genomic alterations in nucleotide sequence that are undetectable by cytogenetics are a crucial aspect of cancer progression, as reviewed by (39). Mutation rate measurements at the *hprt* gene have been widely used in human, Chinese hamster, and other mammalian cells for quantifying mutagenesis in a locus that is responsive to both point mutations and deletions (31, 41, 49, 55). Due to the functionally hemizygous nature of the *hprt* locus [and physical hemizygosity in CHO cells (10)], point mutations, insertions, and gene-size deletions are detectable, but large-scale multigenic deletions and interchromosomal rearrangements are not well tolerated (58). High levels of spontaneous *hprt* mutagenesis are often associated with defects in mismatch repair due to increased tolerance for mis-incorporated nucleotides (11, 15), or with overexpression of translesion polymerases, such as DINB1, Pol  $\kappa$ , and Pol  $\beta$  (2, 3, 32), as such polymerases synthesize DNA in an error-prone manner.

Though of interest, we are not aware of previous studies that have measured spontaneous *hprt* mutation rate in NHEJ deficient cells. HRR-defective *brca2* V-C8 cells were reported to have ~4 fold elevated *hprt* mutation rate compared with the non-isogenic parental hamster V79

line, and the mutant spectrum contained an increased proportion of deletions (24). These results, along with our data, highlight the importance of HRR in preventing loss-of-function mutations that are presumed to arise during DNA replication when broken forks are inaccurately repaired by NHEJ.

In conclusion, the tumorigenic progression of somatic cells toward malignancy depends predominantly on two kinds of altered gene expression: loss of tumor suppressor gene function and gain of inappropriate gene expression (e.g. oncogenes and multi-drug resistance). In this study, we determined the contribution of HRR to two classes of spontaneous gene-specific alterations: (1) loss of *hprt* gene function measured as 6-thioguanine resistance, and (2) *dhfr* and *CAD* gene amplification, which confer drug resistance via increased gene dosage (overexpression). The *rad51d* cells have > 10-fold higher *hprt* mutagenesis and 4- to 10-fold elevated gene amplification rates. For each of the three marker genes, we confirmed the instability phenotype in non-isogenic *xrcc3* cells from the same parental CHO line. These genetic instabilities measured at the single-gene level should be viewed in concert with the high, cytologically measured, chromosomal breakage and exchange, which is elevated by a similar magnitude in *rad51d* cells, but likely has much greater repercussions in terms of lethality.

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Table 1. Spontaneous chromosomal aberrations in *rad51d* mutant and control cell lines<sup>a</sup>

Cell line	No. of cells	Breaks	Gaps	Isochromatid breaks	Asym. & sym. exchanges	Dicentrics	Centric rings
51D1Lox	351	0.07 ± 0.04	0.7 ± 0.1	0.1 ± 0.05	0	0.003	0
51D1.3 (BAC)	350	0.03 ± 0.03	0.8 ± 0.1	0.15 ± 0.07	0	0	0
51D1	353	0.36 ± 0.09	2.2 ± 0.2	0.7 ± 0.1	0.04	0.003	0.01
Fold increase 51D1 vs. 51D1Lox		5.1 <sup>b</sup>	3.4**	6.6**	-	1	-
Fold increase 51D1 vs. 51D1.3		12 <sup>c</sup>	2.8*	4.9*	-	-	-

<sup>a</sup> Values are given as mean number of aberrations per cell ± SEM. Each value is based on the data in three experiments. \* indicates  $P < 0.05$  and \*\* for  $P < 0.01$  using the student t test.

<sup>b</sup>  $P = 0.11$

<sup>c</sup>  $P = 0.09$

Table 2. Distribution of chromosome number in *rad51d* mutant and control cell lines

Cell line	Cell number	Fraction of cells with chromosome number			
		20	21	22	23
51D1Lox	351	$0.09 \pm 0.04$	$0.88 \pm 0.05$	$0.03 \pm 0.01$	$0.003 \pm 0.005$
51D1.3	350	$0.06 \pm 0.01$	$0.91 \pm 0.04$	$0.02 \pm 0.03$	$0.009 \pm 0.008$
51D1	353	$0.06 \pm 0.03$	$0.90 \pm 0.02$	$0.03 \pm 0.04$	$0.006 \pm 0.010$

Table 3. Rate of mutation (gene amplification) to methotrexate resistance (*dhfr* locus) or PALA resistance (*CAD* locus) in *rad51d*, *xrcc3*, *xrcc2*, and control cell lines <sup>a</sup>

Cell line	Methotrexate resistance (units, $\times 10^{-6}$ )		PALA resistance (units, $\times 10^{-6}$ )	
	Mean <sup>b</sup>	SEM	Mean <sup>c</sup>	SEM
AA8	8.8	0.2	7	3
51D1Lox	7	4	10	3
51D1 ( <i>rad51d</i> )	94	4	31	2
51D1.3	5	2	11	2
irs1SF ( <i>xrcc3</i> )	60	10	25	8
1SFwt8	7	3	10 <sup>d</sup>	ND
irs1 ( <i>xrcc2</i> )	ND	ND	18 <sup>d</sup>	ND
GT619	ND	ND	8 <sup>d</sup>	ND

<sup>a</sup> Rates were determined by the method of the mean (Capizzi and Jameson, 1973).

<sup>b</sup> Each experiment was done two or three times with 20 replicate cultures.

<sup>c</sup> Each experiment was done twice with 20 replicates.

<sup>d</sup> Each experiment was done once.

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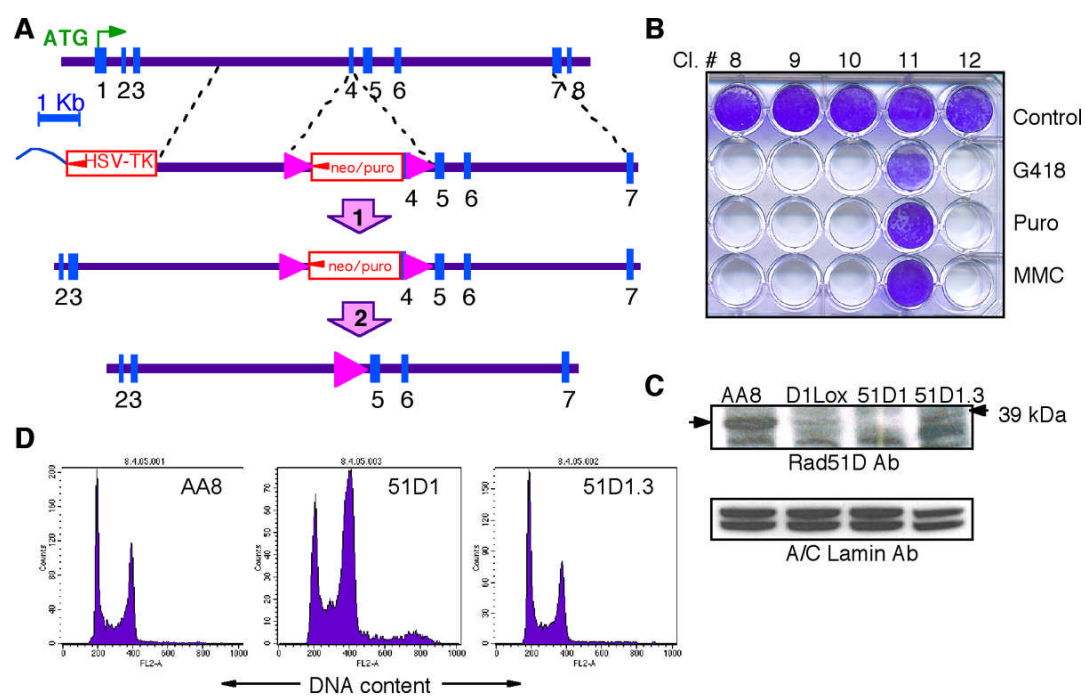
## Figure legends

**FIG. 1.** Targeting vector design and confirmation of targeting. **(A)** Configuration of targeting vectors. The targeting vectors contained a *HSV-TK* gene for negative selection and, for positive selection, a *puro* gene for one allele and a *neo* gene for the other allele. Step 1 results in a targeted recombination event in which exon 4 is replaced by a functional exon 4 flanked by *neo/puro* and LoxP sites. After each allele has been targeted, without affecting *RAD51D* function, treatment with transfected Cre recombinase in step 2 causes deletion of exon 4 and loss of expression of functional Rad51D. **(B)** Screening for exon 4 deletion events in both alleles. After Cre transfection and phenotypic expression, single-cell clones were picked, distributed into 24-well trays, grown for 8 days in media containing, geneticin, puromycin, MMC, or no drug, and then stained. The picture shows a tray in which four of five clones had acquired sensitivity to each selection agent. Some clones (not shown) that were sensitive to only to geneticin or puromycin had presumably experienced recombination in one allele only. **(C)** Western blot of Rad51D protein in parental, mutant, and gene-complemented mutant cells. **(D)** DNA profiles of mutant and control cell lines.

**FIG. 2.** Sensitivity of *rad51d* knockout cells to MMC, IR, MMS, and UV. The AA8 parental cell line was used to create two conditional knockout clones, 51D1Lox and 51D2Lox (e.g. panel B), which have undergone independent gene targeting in the second allele of *RAD51D*. The *rad51d* 51D1 cells were stably transfected with the hamster *RAD51D* gene to produce corrected clones 51D1.3 and 51D1.4 (e.g. panel A). Each data point represents the mean of two or three experiments; error bars are SEM. **(A)** MMC survival curves; note log scale for dose. **(B)**  $\gamma$ -ray survival curves. **(C)** MMS survival curves. **(D)** UV-C survival curves.

**FIG. 3.** Defective Rad51 focus formation and spontaneous chromosomal aberrations in *rad51d* mutant cells. **(A)** Immunofluorescence images of parental and mutant cells exposed to 0 or 8 Gy  $\gamma$ -rays and fixed 4 h after irradiation. **(B)** Percentage of cells having foci ( $\geq 5$ ) after exposure to 8 Gy or 5  $\mu$ M MMS for 1 hr. **(C)** Examples of spontaneous aberrations. IB, isochromatid break; G, gap; AE, asymmetrical exchange; SE, symmetrical exchange, which derives from pairing between homologs. The most common aberrations, single-chromatid breaks, are not seen in this cell.

**FIG. 4.** Mutation rates in *rad51d*, *xrcc3*, and control cell lines. **(A)** Method of the mean calculations. **(B)**  $P_0$  calculation method. AA8 data are taken from (20). Experiments were done three to six times for the Rad51D series and twice for *irs1SF* and *1SFWT8*. Each experiment had 12 or 18 replicate dishes. Error bars are SEM values.



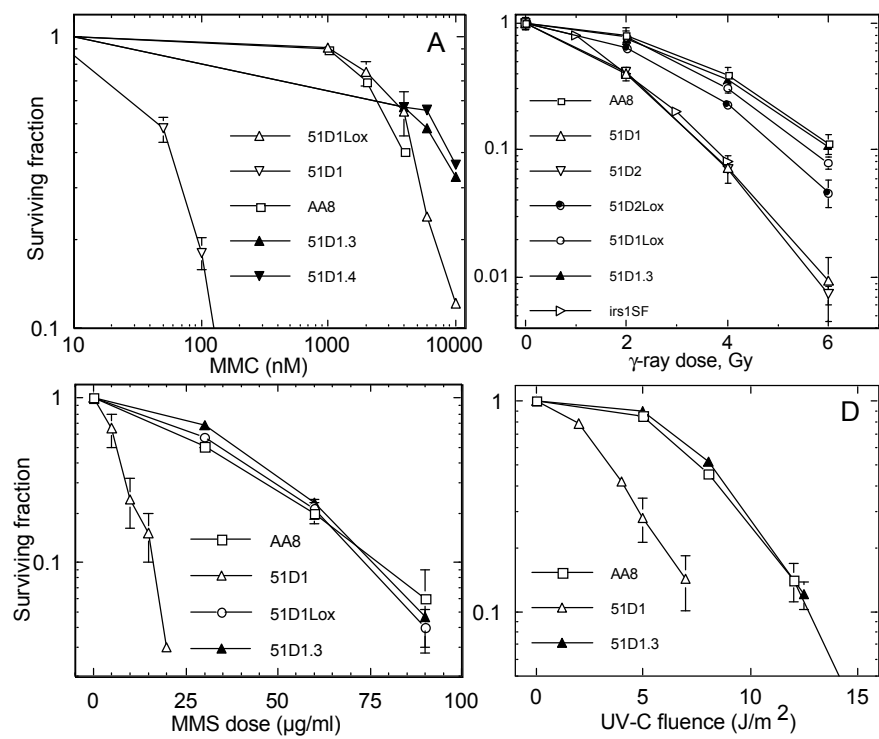


Figure 2

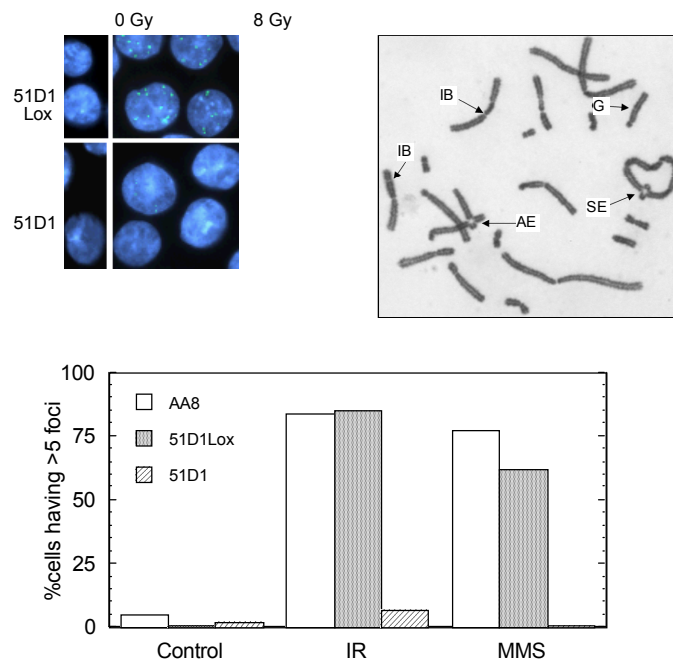


Figure 3

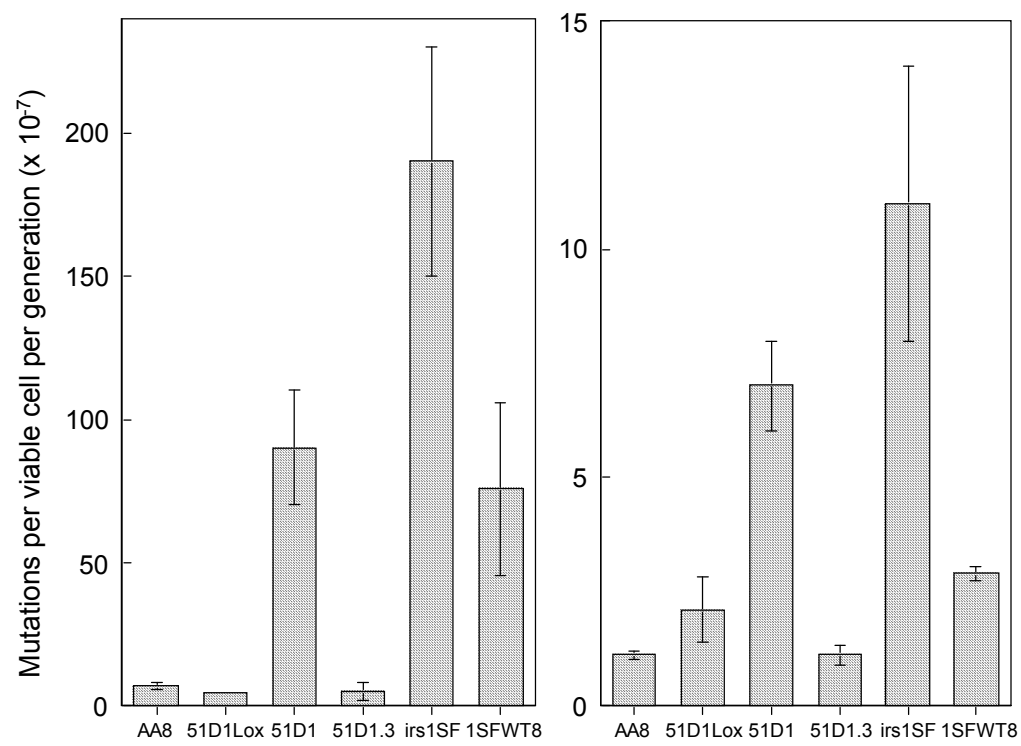


Figure 4